(12) # EUROPEAN PATENT APPLICATION

(54) Selective chemical removal of a protein amino-terminal residue.

(57) The invention relates to a process and compounds useful
therein for producing a compound having an amino acid se-
quence defining a biologically active peptide or protein from a
compound of the formula

H-X-Pro-Peptide

in which X is the residue of a naturally occurring amino acid and
Peptide is a sequence of amino acids defining a biologically ac-
tive peptide or protein , which comprises subjecting H-X-Pro-
Peptide to conditions under which a diketopiperazine of the H-
X-pro- moiety is formed with accompanying cleavage and
release of Peptide.

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SELECTIVE CHEMICAL REMOVAL OF
A PROTEIN AMINO-TERMINAL RESIDUE

The biosynthesis of proteins in genetically
5   altered procaryotic cells results in expression of a
protein having an N-formyl-methionine attached at the
amino terminus. Since the addition of N-formyl-
methionine to the native protein may alter its bio-
logical activity, conformational stability, anti-
10   genicity, etc., it is most desirable, if possible, to
remove it.

By insertion of a cleavage site between the
amino-terminal methionine and the desired native
peptide, one in theory has a greater degree of flexi-
15   bility in the methodology selected for achieving pro-
duction of the desired native peptide. In fact,
however, there are only a very limited number of
practical methods for achieving selective cleavage.

For example, in those native proteins in which
20   methionine is not present, cyanogen bromide mediated
cleavage (methionine being the selective cleavage site)
has proven to be a very effective method for generating
the native protein. In fact, however, the absence of
methionine is a rare occurrence in moderately-sized
25   peptides and proteins. Consequently, a most important
objective is to discover a method by which the amino-
terminal methionine can be cleaved with generation of
the native biosynthetically-produced protein.

A key and unexpected observation determining
30   any approach for generating native protein from amino
terminal methionyl protein is that the α-amino group of

the biosynthetic expression product, for example, N^{α}-
methionyl-(human growth hormone), is not formylated.
This fact was first observed through chemical modifi-
cation of the expression product with cyanate and later
was confirmed by automated Edman sequence analysis. The
E. coli expression organism, although incapable of
cleavage of the initiating methionine, nevertheless, did
effect removal of the N^{α}-formyl group, presumably
enzymatically. Consequently, the α-amino group of the
amino-terminal methionine expression product is directly
available (potentially at least) for cleavage.

Having a free α-amino group on the amino-
terminal methionyl expression product gives rise to the
possibility of other approaches for methionyl removal.
One approach, a selective Edman-type amino terminal se-
quential cleavage using phenyl isothiocyanate, for
practical application requires the absence of lysine
residues in the native protein. However, only the
rarest of proteins will be free of lysine.

Another possible approach is the use of an
exopeptidase; in principle, these will achieve step-
wise amino acid scission. Reports of successful utili-
zation of exopeptidases in synthetic studies have been
quite scarce for a number of reasons, including their
heterogeneous nature, expense, susceptibility to
denaturation, and, most importantly, their wide vari-
ability in specific and non-specific action against
differing substrates.

A new methodology has been discovered which
represents novel chemical procedures and provides new
compounds useful in achieving selective amino-terminal

cleavage to produce the desired end peptide product.
It is to such a process and to compounds useful therein
that this invention is directed.

Thus, this invention relates to a class of
compounds having the formula

H-X-Pro-Peptide

in which X is the residue of a naturally occurring
amino acid; and Peptide is a sequence of amino acid
residues defining that of a biologically active peptide
or protein.

The invention provides a process for producing
a compound having an amino acid sequence defining a
biologically active peptide or protein from a compound
of the formula

H-X-Pro-Peptide

in which X is the residue of a naturally occurring amino
acid and Peptide is a sequence of amino acids defining
a biologically active peptide or protein. In one
embodiment, the process comprises subjecting H-X-Pro-
Peptide to weakly acid conditions in an aprotic solvent.
In a second embodiment of the process H-X-Pro-Peptide is
subjected to acid, neutral, or alkaline conditions in a
buffered aqueous medium. In either embodiment a
diketopiperazine of the H-X-Pro moiety is formed with
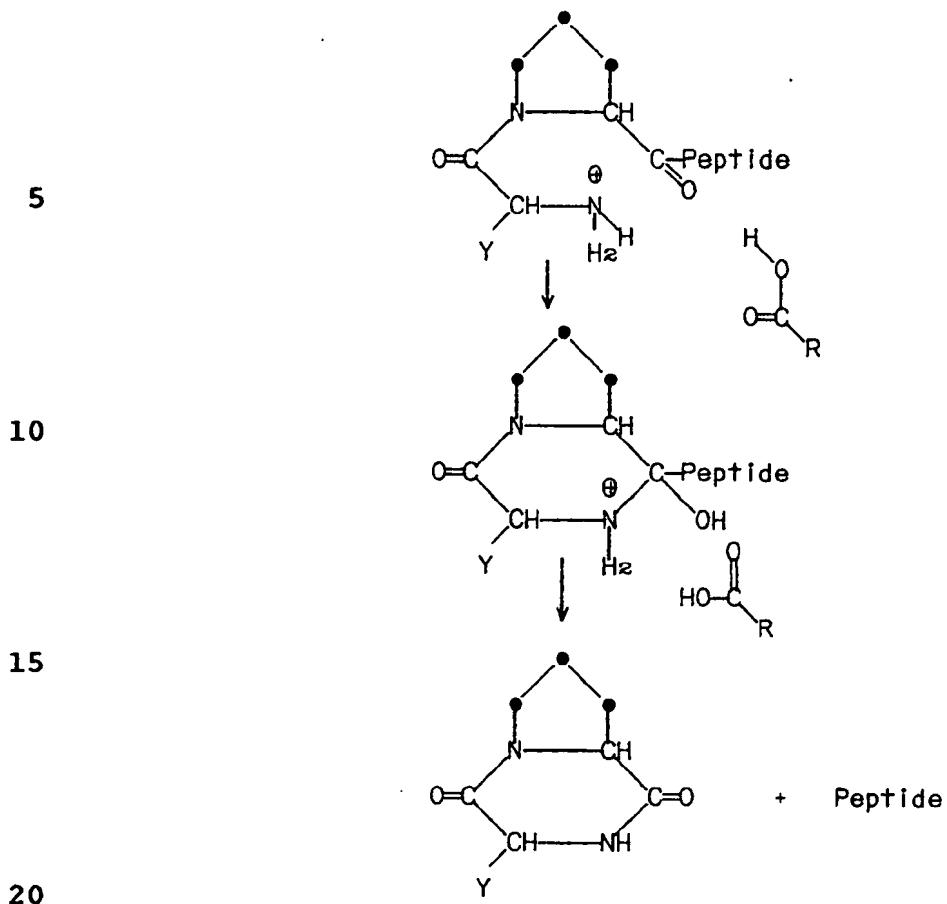accompanying cleavage and release of Peptide.

The methodology that has been discovered and
which represents the substance of this invention
involves the designed biosynthetic production of a
compound of the formula

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H-X-Pro-Peptide

in which X and Peptide have the meanings as herein-
before defined. The compound can be produced using
10   now routine recombinant DNA techniques and can be
obtained either as a direct expression product or
indirectly as a result of treatment of a precursor
expression product.

The compound H-X-Pro-Peptide is extremely
15   useful for obtaining the desired compound designated by
the term "Peptide", which compound could not have been
prepared directly via recombinant DNA methodology. The
compound H-X-Pro-Peptide, when treated in accordance
with the process of this invention, results in formation
20   of the desired product (Peptide) via intermediate
diketopiperazine formation. The cleavage in an aprotic
solvent is generally depicted in the following sequence:

$$\text{(structure 1)}$$

$$\downarrow \quad \underset{O=C}{\overset{H}{\underset{R}{\bigcirc}}}$$

$$\text{(structure 2)}$$

$$\downarrow \quad HO-\underset{R}{\overset{O}{C}}$$

$$\text{(structure 3)} \quad + \quad \text{Peptide}$$

In the foregoing, the group Y is the side chain of a naturally occurring amino acid.

The group X in H-X-Pro-Peptide denotes a residue of any of the naturally occurring amino acids. Preferably, however, the amino acid residue represented by X is other than proline since a Pro-Pro sequence will only very slowly form the desired diketopiperazine intermediate through which the cleavage will proceed. Most often, H-X-Pro-Peptide will be produced via recombinant DNA methodology. When the compound is produced directly as the expression product, the group X

will be methionyl; X, therefore, in the context of this invention, most preferably is methionyl. However, when the group X is other than methionyl, the source of the compound again may be biosynthetic. The expression

5   product, however, will have first been treated by any of a number of methods to produce H-X-Pro-Peptide.

The term "Pro" as used herein refers to the amino acid residue of the naturally occurring amino acid proline.

10   The group designated "Peptide" refers to a sequence of amino acid residues representing that of any of a wide range of biologically active peptides and proteins including, for example, proinsulin, human growth hormone, bovine growth hormone, α-interferon,

15   β-interferon, γ-interferon, interleukin-1, interleukin-2, growth hormone release factor, insulin-like growth factors, tissue plasminogen activator, protein C, and the like, as well as appropriately modified forms of recognized biologically active peptides and proteins.

20   Examples of H-X-Pro-Peptide are:
        Met-Pro-(human growth hormone);
        Ala-Pro-(α-interferon);
        Met-Pro-(interleukin-2);
        Met-Pro-(γ-interferon);

25   Met-Pro-(bovine growth hormone);
        Met-Pro-(tissue plasminogen activator);
        Ser-Pro-(human growth hormone);
        Asp-Pro-(human growth hormone);
        Met-Pro-(growth hormone release factor);

30   Met-Pro-(protein C);
        Leu-Pro-(insulin-like growth factor);
and the like.

Compounds of which the foregoing are exemplary,
structured such that two specifically defined amino acid
residues have been added to the desired end product
peptide, afford generation of a wide range of otherwise
5   unavailable biologically active polypeptides and pro-
teins. The compounds, having at their amino terminus a
dipeptide composed of a natural amino acid residue
followed in sequence by a proline residue, are tailored
to permit ready cleavage of the dipeptide and generation
10   of the desired end product. The penultimate proline
exerts a cis-conformation on the amino terminal dipep-
tide and thereby fixes the terminal α-amino in a more
favored location for attack at the proline carbonyl [see
Gisin, B.F., and Merrifield, R.B., J. Am. Chem. Soc.
15   94, 3102-3106 (1972)]. Nucleophilic attack at this site
is favored by formation of the energetically favored
cyclic amino acid-proline diketopiperazine with release
of the desired peptide as the leaving group. An essen-
tial feature of the reaction in an aprotic solvent is
20   its catalysis by weak acid. A question, key to the
feasibility of that embodiment of the process of this
invention and the answer to which could not be pre-
dicted, is whether the amino-terminal residue of the
desired end product could and would serve as a suitable
25   leaving group so as to depart with diketopiperazine
formation under conditions in which the desired end
product would be both stable and soluble. It appears,
although the volume of published solid phase peptide
synthesis literature is vast, that diketopiperazine
30   formation has only been reported at the dipeptidyl
stage of synthesis where hydrolysis is of the function-

alized resin ester linkage. The absence of any reports
of diketopiperazine formation at X-Pro sequences where
amide hydrolysis is required is not surprising due to
the generally accepted principle of increased acid

5   stability of amides when compared to esters. This
silence argues strongly against the potential success
of the process of this invention.

        The treatment of the H-X-Pro-Peptide compound
in accordance with the first embodiment of the process

10 of this invention is carried out in an aprotic solvent,
preferably substantially free of water. In general,
that condition can be met merely by avoiding any affirm-
ative addition of water. Examples of aprotic solvents
are 1-methylpyrrolidinone (NMP), N,N-dimethylformamide

15 (DMF), dimethyl sulfoxide (DMSO), formamide, hexa-
methylphosphoramide (HMPA), and the like. Preferred
solvents are NMP, DMF, and DMSO, and, of these, NMP is
most preferred.

        The reaction in the aprotic solvent is con-

20 ducted under weakly acidic conditions using acetic acid,
phosphoric acid, phosphorous acid, sulfuric acid,
trifluoroacetic acid, formic acid, maleic acid, tartaric
acid, glycine, and the like. A highly preferred acid
is acetic acid.

25   The cleavage reaction also can be conducted
in buffered aqueous medium. When water is the reaction
medium, it is preferred to conduct the reaction in a
phosphate buffer.

        When conducted in an aprotic solvent, the

30 process of this invention generally will be carried out
at a temperature within the range of from about 15°C

to about 50°C, preferably from about 25°C to about
40°C. The acid concentration usually will range from
about 0.1$\underline{M}$ to about 1$\underline{M}$, preferably from about 0.25$\underline{M}$
to about 0.5$\underline{M}$, most preferably from about 0.30$\underline{M}$ to
5   about 0.35$\underline{M}$, and specifically about 0.33$\underline{M}$.

When conducted in a aqueous medium, the
temperature needs to be somewhat higher. Thus, for
example, the temperature generally will range from about
25°C to about 60°C, preferably from about 35°C to about
10   50°C. The choice of reaction temperature is dependent
on the stability of "Peptide" and the nature of the
third amino acid in H-X-Pro-Peptide. As high a tem-
perature as possible, consistent with stability and the
nature of the third amino acid residue, is preferred.
15   The buffer concentration usually will range from about
0.1$\underline{M}$ to about 2$\underline{M}$, preferably from about 0.5$\underline{M}$ to about
1$\underline{M}$, and most preferably from about 0.8$\underline{M}$ to about 1$\underline{M}$.
The preferred buffering salt is phosphate. A pH of
about 8 is preferred as the yield of cleavage is only
20   slightly increased at higher pH values and the pos-
sibility of undesirable side effects is greater at the
higher values [see McKerrow, J.H., and Robinson, A.B.,
Analytical Biochem. 42, 565-568 (1972)].

The cleavage reaction is conducted with
25   periodic monitoring of diketopiperazine formation.
The reaction is terminated when deemed appropriate, and
the cleavage product is recovered from the reaction
mixture using recognized isolation methodology.

The following is provided as examples to
30   illustrate this invention. The examples are not
intended to be limiting on the broad scope of the
invention.

Selected peptides, useful as models for the
present invention, were prepared by solid phase syn-
thetic methodology. Their purity was confirmed by high
performance liquid chromatography. Prior to cleavage,
5   the peptides were stored as amorphous lyophilized
solids. A number of experimental parameters were
varied. Their effect on cleavage is reported herein.

The following describes the optimized condi-
tions using organic and aqueous media, respectively, and
10   applied to the peptide Met-Pro-Gly-Gly-NH$_2$, in which
"-NH$_2$" signifies the presence of an amide moiety at
the C-terminus of the peptide.

A.   Organic Cleavage Conditions
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Ten milligrams of Met-Pro-Gly-Gly-NH$_2$ were
dissolved in 1 ml of N-methylpyrrolidinone which had
been made anhydrous by storage over 4Å molecular sieves
for one week. The mixture was maintained at 25°C with
20   constant stirring, and the reaction was initiated by
addition of glacial acetic acid (HOAc) to a concen-
tration of 0.33$\underline{M}$. The rate of cleavage was monitored
by appearance of methionyl-proline diketopiperazine
formation and disappearance of starting peptide as
25   measured in reverse phase chromatographic analysis.
Chromatography was achieved using a 0.46 x 25 cm column
of Ultrosphere C$_{18}$ in 0.1% trifluoroacetic acid (TFA),
using an acetonitrile gradient to effect elution. The
reaction was stopped at each analysis time point by
30   ten-fold dilution with 0.1% trifluoroacetic acid. The
diketopiperazine peak was collected and its identity
confirmed by amino acid and mass spectral analysis.

The following Tables 1-8 provide results obtained using organic cleavage conditions but varying selected parameters.

Tables 1 reports the level of diketopiperazine
5   formation at varying acid concentrations when the cleavage is carried out at 25°C in DMF. An appreciable rate of reaction is recorded, and it is observed to be dependent upon weak acid concentration. Similar weak acid treatment in DMSO revealed a reduced rate of
10 cleavage. Increasing the temperature of cleavage to 40°C in DMSO revealed comparable rates of reaction to those achieved in DMF at 25°C, as shown in Table 2.

Table 1

Acid Concentration

(Methionine-Proline Diketopiperazine Formation
from Met-Pro-Gly-Gly-NH$_2$ at 25°C)

	Reaction Time, Hours		
Reagent	2	4	6
10 M HOAc/DMF	2%	8%	40%
1 M HOAc/DMF	26%	44%	55%
0.1 M HOAc/DMF	13%	28%	34%
0.01 M HOAc/DMF	3%	6%	8%
100% DMF	0%	3%	4%
100% HOAc	<1%	<1%	2%
100% TFA	0%	<1%	<1%

Table 2

Acid Concentration

5

(Methionine-Proline Diketopiperazine Formation
from Met-Pro-Gly-Gly-NH$_2$ at 40°C)

Reagent	Reaction Time, Hours		
	2	4	24
0.1 M HOAc/DMSO	25%	45%	95%
0.33 M HOAc/DMSO	38%	61%	100%
1.0 M HOAc/DMSO	27%	44%	94%
3.3 M HOAc/DMSO	7%	12%	41%
10.0 M HOAc/DMSO	2%	4%	14%

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As the foregoing demonstrates, the apparent
optimal acid concentration is about 0.25M to about 0.5M.

Table 3 provides results studying the effect

20 . of temperature on a series of peptides in which only the
side chain of the third residue is varied. It is quite
clear that increased steric hindrance at this site will
slow the rate of reaction. However, in all cases,
appreciable cleavage was noted.

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Table 3

Temperature Effect

(Methionine-Proline Diketopiperazine Formation
in 1M Acetic Acid/DMF at Two Temperatures)

Peptide[b]	Reaction Time at 25°C, Hours		Reaction Time at 40°C, Hours	
	2	24	2	24
MPGG-NH$_2$	27%	100%	76%	100%
MPTG-NH$_2$	N.D.[a]	N.D.	22%	88%
MPPG-NH$_2$	N.D.	N.D.	20%	95%
MPFG-NH$_2$	2%	17%	10%	68%

[a] N.D., Not Determined

[b] MPGG-NH$_2$ = Met-Pro-Gly-Gly-NH$_2$
MPTG-NH$_2$ = Met-Pro-Thr-Gly-NH$_2$
MPPG-NH$_2$ = Met-Pro-Pro-Gly-NH$_2$
MPFG-NH$_2$ = Met-Pro-Phe-Gly-NH$_2$

An important factor is the choice of the
aprotic solvent. The solvent must be one which promotes
or at least does not inhibit diketopiperazine formation
with accompanying cleavage to produce the desired end
product while at the same time having minimal degrada-
tive effect on the ultimate peptide product. Tables 4-8
provide results from studies designed to elicit infor-
mation as to the properties of a variety of solvents for
use under organic cleavage conditions.

Table 4 provides stability information of a
number of proteins and peptides when subjected to
organic cleavage conditions. The amount in percent, as
determined by reverse phase high performance liquid

chromatography (HPLC), remaining after subjecting the selected protein or peptide to the defined reaction medium for the stated time in hours is provided and shows, as between DMF and DMSO, the relative superiority of DMSO.

Table 4

Protein Stability

Protein[a]	1M HOAc/DMF, 40°C			1M HOAc/DMSO, 40°C		
	2h	24h	48h	2h	24h	48h
Glucagon	100%[b]	49%	37%	124%	117%	119%
bPP	42%	40%	32%	52%	90%	86%
GRF	79%	26%	0%	98%	89%	86%
$A(SO_3^-)_4$	100%	58%	36%	100%	100%	96%
$B(SO_3^-)_2$	97%	62%	37%	94%	106%	68%
Proinsulin	86%	43%	30%	97%	70%	86%

[a] bPP = bovine pancreatic polypeptide

GRF = growth hormone release factor

$A(SO_3^-)_4$ = insulin A-chain S-sulfonate

$B(SO_3^-)_4$ = insulin B-chain S-sulfonate

[b] The relative percent remaining is determined against an untreated external control.

Table 5, similar to Table 4, provides stability results using methionyl human growth hormone, in which NMP proved to be more nearly like DMSO than DMF.

Table 5

Stability of Methionyl Human Growth Hormone

Reagent	Reaction Time at 40°C, Hours		
	2	4	24
0.33 M HOAc/DMF	78[a]/78[b]	65/68	45/34
0.33 M HOAc/DMSO	91/100	77/99	75/86
0.33 M HOAc/NMP	85/90	70/89	77/75

[a]Determined by reverse phase HPLC
[b]Determined by anion exchange HPLC

Table 6 elaborates the results studying the effect of a number of solvents on cleavage of Met-Pro-Gly-Gly-NH$_2$.

Table 6

Solvent Effect

(Disappearance of Met-Pro-Gly-Gly-NH$_2$ as a
Function of Met-Pro Diketopiperazine Formation)

Reagent	Reaction Time at 25°C, Hours				
	0	2	4	6	24
1M HOAc/CH$_3$CN	100%[a]	114%	105%	99%	66%
1M HOAc/nPrOH	N.D.[b]	100%	97%	94%	72%
1M HOAc/MeOH	100%	101%	99%	99%	97%
1M HOAc/CH$_2$Cl$_2$	100%	104%	96%	99%	80%
1M HOAc/DMF	100%	88%	71%	57%	11%
1M HOAc/DMSO[c]	100%	73%	56%	N.D.[a]	6%

[a] Represents the quantity of starting material remaining.
[b] N.D., Not Determined
[c] Determined at 40°C.

From the foregoing, the highly preferred
solvents are NMP, DMF, and DMSO, all aprotic organic
solvents.

Table 7 provides results studying the effect
of temperature on a variety of peptides using DMF or
DMSO as solvent.

Table 7

Temperature Effect

(Methionine-Proline Diketopiperazine Formation
in two selected solvents at 1$\underline{M}$ HOAc, 40°C)

	DMF (Time in Hours)		DMSO (Time in Hours)		
Peptide[a]	2	24	2	24	48
MPGG-NH$_2$	76%	100%	36%	100%	100%
MPTG-NH$_2$	22%	88%	3%	11%	38%
MPPG-NH$_2$	20%	95%	5%	43%	65%
MPFG-NH$_2$	10%	68%	1%	12%	23%

[a] MPGG-NH$_2$ = Met-Pro-Gly-Gly-NH$_2$
MPTG-NH$_2$ = Met-Pro-Thr-Gly-NH$_2$
MPPG-NH$_2$ = Met-Pro-Pro-Gly-NH$_2$
MPFG-NH$_2$ = Met-Pro-Phe-Gly-NH$_2$

As the data from Table 7 and the foregoing
other tables show, DMF relative to DMSO has a greater
effect on diketopiperazine formation and accompanying
cleavage; however, it also has a greater degrading
effect on the product protein or peptide. A fine
balance in selection of reaction conditions with respect
to any particular peptide or protein product must be
determined and applied for achieving optimal results.

Table 8 presents data resulting from the
application of organic cleavage conditions to a model
peptide that mimics a compound of this invention in
which the group Peptide is human growth hormone. It is
noteworthy that human growth hormone presents a special
situation since the first two amino acids of human

growth hormone are Phe-Pro. Thus, it is possible to
. form the diketopiperazine from X-Pro of a compound of
this invention and to find a succeeding diketopiperazine
form from the initiating Phe-Pro of the resulting human

5   growth hormone. Table 8 thus compares Met-Pro-Phe-Pro-
Thr-Ile-NH$_2$ with Phe-Pro-Thr-Ile-NH$_2$. The results show
a slower diketopiperazine formation from the latter.
Thus, the conditions of treatment of the compound
Met-Pro-human growth hormone, a compound of this

10 invention, can be precisely determined so as to maximize
the single diketopiperazine formation and accompanying
cleavage with minimal secondary diketopiperazine
formation. In this regard, NMP is a most desirable
solvent since the level of diketopiperazine formation is

15 significantly greater than that in DMSO and almost
matches that achieved in DMF, at later time points.
When considering the increased stability of growth
hormone to NMP than to DMF (Table 5), the former clearly
is the desired solvent, at least for this application.

Table 8

Diketopiperazine Formation from Growth Hormone Model Peptides

$Met-Pro-Phe-Pro-Thr-Ile-NH_2$

Reagent	Reaction Time at 40°C, Hours			
	2	4	6	24
0.33$\underline{M}$ HOAc/NMP	17%	32%	47%	99%
0.33$\underline{M}$ HOAc/DMSO	4%	6%	27%	33%
0.33$\underline{M}$ HOAc/DMF	14%	47%	63%	98%

$Phe-Pro-Thr-Ile-NH_2$

Reagent	Reaction Time at 40°C, Hours			
	2	4	6	24
0.33$\underline{M}$ HOAc/NMP	7%	12%	23%	78%
0.33$\underline{M}$ HOAc/DMSO	4%	7%	11%	27%
0.33$\underline{M}$ HOAc/DMF	15%	26%	37%	88%

B. Aqueous Cleavage Conditions

Optimal aqueous cleavage of $Met-Pro-Gly-Gly-NH_2$ was achieved by dissolving 1 mg of the peptide in 1$\underline{M}$ sodium phosphate buffer, pH 8.0. To accelerate the cleavage, the temperature was maintained at 55°C for 24 hours. The degree of cleavage was determined in the manner described for organic cleavage.

The following Tables 9 to 12 provide results obtained using acid, neutral or alkaline conditions in a buffered aqueous medium.

Table 9 reports the cleavage yield at pH 7.0 using the peptide Met-Pro-Gly-Gly-NH$_2$ as substrate. Three different temperatures were examined with two different buffering salts and concentrations. The results show that phosphate buffer is to be preferred over acetate with a concentration of 1.0M providing the best results.

Table 9

Effect of Different Buffer Systems
and Concentrations on Methionine-Proline
Diketopiperazine Formation from
Met-Pro-Gly-Gly-NH$_2$ at pH 7.0

	Formation*		
Buffer	100°C	40°C	25°C
0.1M Sodium Phosphate	100%	70%	14%
1.0M Sodium Phosphate	100%	93%	25%
0.1M Sodium Acetate	55%	12%	1%
1.0M Sodium Acetate	86%	35%	4%

*Methionine-proline diketopiperazine formation at
 100°C was measured at 2 hours. Formation at 40°C
 and 25°C was measured at 72 hours.

A study of the effect of pH on methione-proline diketopiperazine formation from Met-Pro-Gly-Gly-NH$_2$ at 40°C in buffered aqueous medium is reported in Table 10. Sodium phosphate was used as the buffer salt. The results show that an alkaline pH is to be preferred. As undesirable side reactions, such as deamidation and desulfurization, are accelerated at elevated pH values, a pH of about 8 is preferred.

Table 10

Effect of pH on Methionine-Proline
Diketopiperazine Formation in 1.0M
Sodium Phosphate at 40°C

	Time in Hours			
	4	24	48	72
pH 3	1%	30%	31%	9%
pH 5	2%	9%	18%	25%
pH 7	18%	29%	89%	92%
pH 8	27%	80%	97%	100%
pH 9	30%	86%	98%	100%

Table 11 reports the results of an examination
of the effect of different buffering salts on the
cleavage yield from Met-Pro-Gly-Gly-NH$_2$ at pH 8.0 and
40°C. Sodium phosphate is the preferred buffer.

Table 11

Effect of Different Buffering Salts on
Methionine-Proline Diketopiperazine Formation
from Met-Pro-Gly-Gly-NH$_2$ at pH 8.0 and 40°C

	Time in Hours	
Buffer	2	24
1.0M Sodium Phosphate	22%	86%
1.0M Sodium Phosphite	6%	40%
1.0M Sodium Sulfite	6%	35%

Table 12 reports the results of a study of
the effect of temperature on methionine-proline diketo-
piperazine formation in a series of peptides in which
only the side chain of the third residue from the N-
5   teraminus is varied. As was the case in the study
reported in Table 3, _supra_, it is clear that increased
steric hindrance at the site of the third residue will
slow the reaction. However, appreciable cleavage was
noted in all but two instances. Increasing the tem-
10   perature from 40°C to 55°C markedly increased the rate
and extent of cleavage in all four of the tetrapeptides
tested.

Table 12

15

Temperature Effect

(Methionine-Proline Diketopiperazine Formation
at Two Temperatures in 1.0M Phosphate
20   Buffer at pH 8.0

Peptide[a]	Reaction Time at 40°C, Hours			Reaction Time at 55°C, Hours		
	2	24	48	2	24	48
MPGG-NH$_2$	16%	84%	96%	47%	100%	100%
MPTG-NH$_2$	2%	18%	26%	8%	47%	75%
MPPG-NH$_2$	0%	12%	19%	6%	30%	53%
MPFG-NH$_2$	0%	9%	14%	4%	28%	57%

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[a] MPGG-NH$_2$ = Met-Pro-Gly-Gly-NH$_2$
MPTG-NH$_2$ = Met-Pro-Thr-Gly-NH$_2$
MPPG-NH$_2$ = Met-Pro-Pro-Gly-NH$_2$
MPFG-NH$_2$ = Met-Pro-Phe-Gly-NH$_2$

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Analysis of the products obtained via the
foregoing cleavage methods upon a representative sampling
of peptides and proteins was assessed by high performance
anion exchange and reverse phase chromatography. Anion
5   exchange chromatography was conducted on a Mono Q column
in a 0.05$\underline{M}$ Tris, pH 8.0 buffer containing 30% acetonitrile.
Elution was provided using a linear gradient of sodium
chloride. Reverse phase analysis was performed on a
Zorbax C_8, 150Å pore size column using an acetonitrile
10   gradient in 0.1$\underline{M}$ ammonium phosphate at 45°C, pH 7.0.

CLAIMS

1.   A compound having the formula

H-X-Pro-Peptide

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in which X is the residue of a naturally occurring amino
acid; and Peptide is a sequence of amino acid residues
defining that of a biologically active peptide or protein.
2.   The compound of Claim 1 in which X is Met.
10   3.   The compound of Claim 1 or 2 in which
Peptide is a sequence of amino acid residues defining a
peptide selected from the group consisting of pro-
insulin, human growth hormone, bovine growth hormone,
α-interferon, β-interferon, γ-interferon, interleukin-1,
15   interleukin-2, growth hormone release factor, insulin-
like growth factors, tissue plasminogen activator,
protein C, and modified, biologically active forms of
any of the foregoing.
             4.   The compound of Claim 3 in which Peptide
20   is human growth hormone.
             5.   The compound of Claim 3 in which Peptide
is bovine growth hormone.
             6.   A process for producing a compound having
an amino acid sequence defining a biological active
25   peptide or protein from a compound of the formula
                     H-X-Pro-Peptide
wherein X is the residue of a naturally occurring amino
acid and Peptide is a sequence of amino acids defining
a biologically active peptide or protein which comprises
30   subjecting H-X-Pro-Peptide to

a)   weakly acid conditions in an aprotic solvent,
      or

b)   acid, neutral or alkaline conditions in a
      buffered aqueous medium,

to form a diketopiperazine of the H-X-Pro moiety with
accompanying cleavage and release of Peptide.

        7.   The process of Claim 6 in which X is Met.

        8.   The process of Claim 6 or 7 in which
Peptide is a sequence of amino acid residues defining
proinsulin, human growth hormone, bovine growth hormone,
α-interferon, β-interferon, γ-interferon, interleukin-1,
interleukin-2, growth hormone release factor, insulin-
like growth factors, tissue plasminogen activator,
protein C, or modified, biologically active forms of any
of the foregoing.

        9.   The process of Claim 8 in which Peptide is
human growth hormone.

        10. The process of Claim 8 in which Peptide is
bovine growth hormone.

        11. The process of any of Claims 6 to 10 in
which the aprotic solvent is N-methylpyrrolidinone,
N,N-dimethylformamide, dimethyl sulfoxide, formamide, or
hexamethylphosphoramide.

        12. The process of Claim 11 in which the
aprotic solvent is N-methylpyrrolidinone.

        13. The process of any of Claims 6 to 12 in
which the weakly acid conditions are generated by
addition of acetic acid, phosphoric acid, phosphorous
acid, sulfuric acid, trifluoroacetic acid, formic acid,
maleic acid, tartaric acid, or glycine.

14. The process of Claim 13 in which the acid is acetic acid.

15. The process of any of Claims 6 to 14 which is carried out at a temperature of from about 15°C to about 50°C.

16. The process of any of Claims 6 to 15 in which the acid concentration is from about 0.1$\underline{M}$ to about 1$\underline{M}$.

17. The process of Claim 16 in which the acid concentration is from about 0.30$\underline{M}$ to about 0.35$\underline{M}$.

18. The process of any of Claims 6 to 10 which comprises subjecting H-X-Pro-Peptide to neutral or alkaline conditions in a buffered aqueous medium.

19. The process of Claim 18 in which the buffering salt is a phosphate, phosphite, or sulfite salt.

20. The process of Claim 19 in which the buffering salt is a phosphate salt present in a concentration of from about 0.1$\underline{M}$ to about 2.0$\underline{M}$.

21. The process of Claim 20 in which the phosphate salt is present in a concentration of about 0.8$\underline{M}$ to about 1.0$\underline{M}$.

22. The process of any of Claims 18 to 21 which is carried out at a pH of from about 7 to about 9.

23. The process of Claim 22 which is carried out at about pH 8.

24. The process of any of Claims 18 to 23 which is carried out at a temperature of from about 25°C to about 60°C.

25. The process of Claim 24 which is carried out at a temperature of from about 35°C to about 50°C.

CLAIMS

1. A process for producing a compound having an amino acid sequence defining a biologically active peptide or protein from a compound of the formula

                    H-X-Pro-Peptide

wherein X is the residue of a naturally occurring amino acid and Peptide is a sequence of amino acids defining a biologically active peptide or protein; characterized by subjecting H-X-Pro-Peptide to

   a)   weakly acid conditions in an aprotic solvent, or

   b)   acid, neutral or alkaline conditions in a buffered aqueous medium,

to form a diketopiperazine of the H-X-Pro moiety with accompanying cleavage and release of Peptide.

2. The process of Claim 1, characterized in that the aprotic solvent is N-methylpyrrolidinone, N,N-dimethylformamide, dimethyl sulfoxide, formamide, or hexamethylphosphoramide.

3. The process of Claim 2, characterized in that the aprotic solvent is N-methylpyrrolidinone.

4. The process of Claim 1, 2 or 3 characterized in that the weakly acid conditions are generated by addition of acetic acid, phosphoric acid, phosphorous acid, sulfuric acid, trifluoroacetic acid, formic acid, maleic acid, tartaric acid, or glycine.

5. The process of Claim 4, characterized in that the acid is acetic acid.

6. The process of any of Claims 1 to 5 characterized in that the reaction is carried out at a temperature of from about 15°C to about 50°C.

7. The process of any of Claims 1 to 6, characterized in that the acid concentration if from about 0.1$\underline{M}$ to about 1$\underline{M}$.

8. The process of Claim 7, characterized in that the reaction is carried out using acetic acid at a concentration of about 0.33$\underline{M}$.

9. The process of Claim 1, characterized by subjecting H-X-Pro-Peptide to neutral or alkaline conditions in a buffered aqueous medium.

10. The process of Claim 9, characterized in that the buffering salt is a phosphate, phosphite, or sulfite salt.

11. The process of Claim 10, characterized in that the buffering salt is a phosphate salt present in a concentration of from about 0.1$\underline{M}$ to about 2.0$\underline{M}$.

12. The process of Claim 11, characterized in that the phosphate concentration is from about 0.8$\underline{M}$ to about 1$\underline{M}$.

13. The process of any of Claims 1 or 9 to 12, characterized in that the process is carried out at a pH of from about 7 to about 9.

14. The process of Claim 13, characterized in that the process is carried out at a pH of about 8.

15. The process of any of Claims 1 or 9 to 13, characterized in that the process is carried out at a temperature of from about 25°C to about 60°C.

16. The process of any of Claims 1 to 15, characterized in that X is Met.

17. The process of any of Claims 1 to 16, characterized in that Peptide is a sequence of amino acid residues defining proinsulin, human growth hormone,

bovine growth hormone, α-interferon, β-interferon, γ-interferon, interleukin-1, interleukin-2, growth hormone release factor, insulin-like growth factors, tissue plasminogen activator, protein C, or modified,

5   biologically active forms of any of the foregoing.

18. The process of Claim 17, characterized in that Peptide is human growth hormone.

19. The process of Claim 17, characterized in that Peptide is bovine growth hormone.